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Communication to the Editor

Degradation of Bromoxynil, Ioxynil, Dichlobenil and their Mixtures by *Agrobacterium radiobacter* 8/4

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Abstract: Degradation of three benzonitrile herbicides, bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), ioxynil (3,5-diiodo-4-hydroxybenzonitrile), dichlobenil (2,6-dichlorobenzonitrile), and their mixtures by the soil micro-organism *Agrobacterium radiobacter* 8/4 was studied in batch cultures. Bromoxynil was found to be most rapidly degraded, while dichlobenil had the lowest toxicity to our strain. All transformations of studied benzonitriles were performed by the nitrile hydratase which has been shown to act on a broad range of substituted aromatic nitriles.

Key words: biodegradation, *Agrobacterium*, bromoxynil, ioxynil, dichlobenil, herbicides

1 INTRODUCTION

In the last 30 years a large variety of recalcitrant halogenated aromatic compounds have been released into the environment for different purposes. Bromoxynil, ioxynil and dichlobenil are widely used benzonitrile herbicides. Bromoxynil and ioxynil are often applied as post-emergence herbicides for crop protection, while dichlobenil is used both pre-emergence and post-emergence. They are also often applied in mixtures with other herbicides.[1]

Although numerous micro-organisms utilize aliphatic nitriles,[2–4] there is still only a limited number of reports concerning the degradation of halogenated aromatic nitriles[5–8] and none of them addresses the potential for mutual inhibitory effects of commonly used herbicides on strains responsible for their degradation. In our opinion this is an important aspect since herbicide products usually contain mixtures of several active ingredients. Hence, in this study we have focused on the growth and degradative activities of the strain *Agrobacterium radiobacter* 8/4[9] in the presence of individual herbicides, or their mixtures, respectively. The strain was selected due to its ability to transform a wide range of commercially used aromatic nitriles by the action of nitrile hydratase.

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2 EXPERIMENTAL

2.1 Chemicals

Bromoxynil was kindly provided by Aldrich, ioxynil, dichlobenil and 2,6-dichlorobenzamide by Riedel de Haen. 3,5-Dibromo- and 3,5-diiodo-4-hydroxybenzamide were prepared by hydration of bromoxynil and ioxynil,[10] respectively, and were subsequently purified by thin-layer chromatography. All other chemicals were of analytical grade and available commercially.

2.2 Media, cultures and growth conditions

The enrichment isolation of *A. radiobacter* 8/4 stain from bromoxynil-treated soil has been already described.[9]

A. radiobacter 8/4 cells were grown in batch culture on a mineral salt medium[11] containing D-glucose (1 g litre^{-1}) and 50 mg litre^{-1} of tested herbicide at 28°C.

Bromoxynil, ioxynil and dichlobenil were added from a dimethyl sulfoxide stock solution (50 g litre^{-1}) to a final concentration of 50 mg litre^{-1} before sterilization.

Samples for HPLC analysis and growth curves were taken at 8-h intervals. The optical density of the culture was measured at 600 nm on a UV/VIS spectrophotometer SP6-555 (Pye-Unicam).

2.3 HPLC

Bromoxynil, ioxynil, dichlobenil and their metabolites were separated and determined by HPLC as described elsewhere.[12]

2.4 Enzyme assays

A. radiobacter 8/4 cells were cultured on LB medium overnight, harvested by centrifugation, and washed with phosphate buffer (0·1 M; pH 7·0). The cells were then resuspended in the same buffer, disrupted by French-press, and the extract was centrifuged for 30 min at 20 000 *g*. 500 μl of the cell-free extract was mixed with 4·5 ml of the phosphate buffer supplied with the test nitriles (final concentration 50 mg litre^{-1}) and incubated at 28°C. Samples were taken at 1, 2 and 4 h, respectively. Protein was determined by the method of Bradford[13] with BSA as a standard. All results were the means of three experiments.

3 RESULTS AND DISCUSSION

3.1 Influence of herbicides on growth of *Agrobacterium radiobacter* 8/4

The cell density of the culture was monitored and correlated to the amount of herbicide degradation. From the growth curves of bacteria in a medium with a single herbicide it can be seen that all tested herbicides prolonged the lag phase and decreased the final optical density of the culture (Fig. 1). Bromoxynil and ioxynil were more inhibitory to growth than dichlobenil.

The situation with the growth inhibition of *A. radiobacter* 8/4 by the herbicide mixtures, however, was quite different when the most inhibiting two-compound mixture was that of ioxynil with dichlobenil (Fig. 2). Almost the same level of inhibition was observed in a mixture of all the three studied herbicides.

3.2 *Agrobacterium radiobacter* 8/4 degradation of herbicides and their mixtures

The ability of *A. radiobacter* 8/4 to degrade bromoxynil, ioxynil and dichlobenil applied individually or in a mixture is shown in Fig. 3. Individually, bromoxynil was degraded at the highest rate, ioxynil was degraded to a lesser extent, and dichlobenil least.

Bromoxynil was also degraded most rapidly in all mixtures, while ioxynil was better degraded in the presence of dichlobenil than in the presence of bromoxynil. Dichlobenil was also degraded more rapidly in the presence of ioxynil than in the presence of bromoxynil, while the most rapid degradation of this substrate

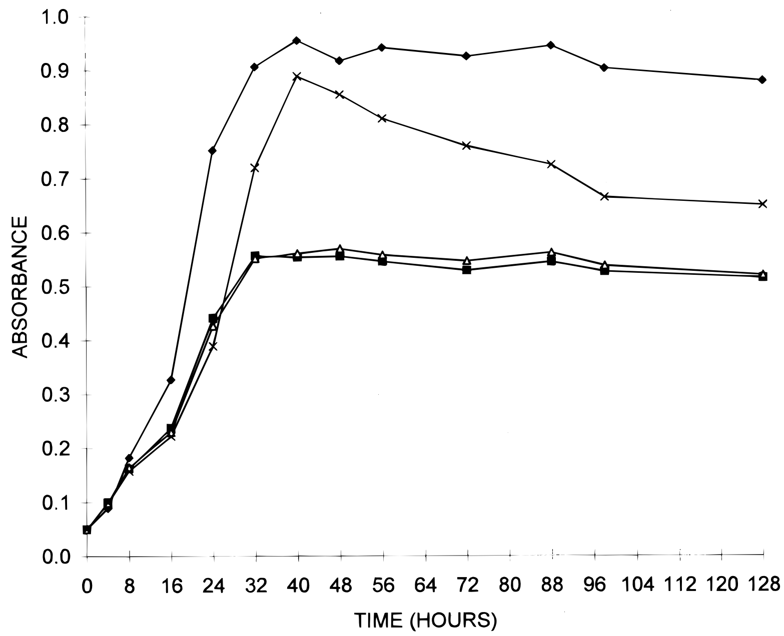

Fig. 1. Growth curve of *Agrobacterium radiobacter* 8/4 in the presence of single herbicides. (◆) control (■) bromoxynil (△) ioxynil (×) dichlobenil.

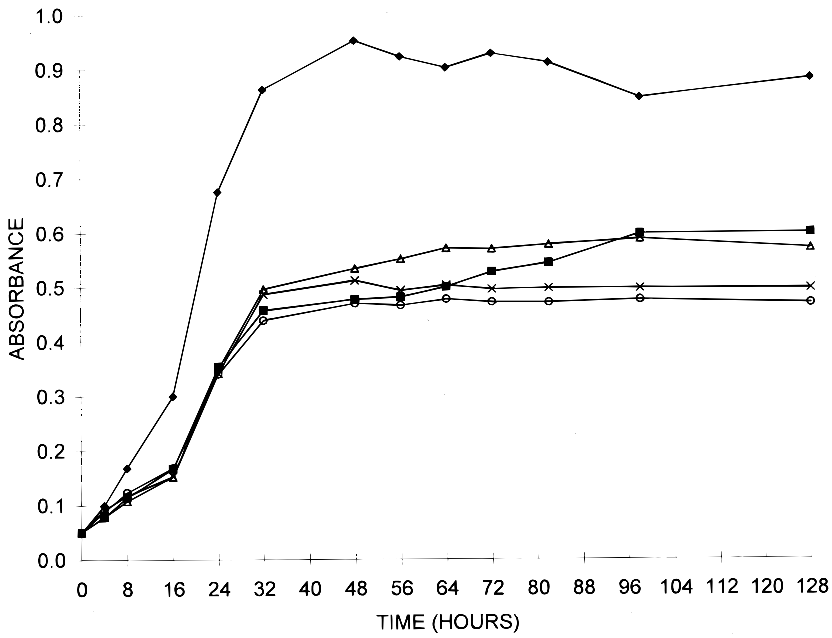

Fig. 2. Growth curve of *Agrobacterium radiobacter* 8/4 in the presence of herbicide mixtures. (◆) control (■) bromoxynil + ioxynil (△) bromoxynil + dichlobenil (×) ioxynil + dichlobenil (○) bromoxynil + ioxynil + dichlobenil.

occurred in the mixture of all three herbicides. As the dichlobenil degradation individually was relatively low, it is possible, that ioxynil, and to a lesser degree bromoxynil, serve as inducers of nitrile hydratase for dichlobenil degradation. This would not be surprising since such enzyme induction by these types of substrate analogue has often been described in the literature. For example, Stokes *et al.* reported a stimulation of polychlorinated biphenyl degradation by bromobenzoic acid.[14] However, we cannot exclude potential induction of other discrete enzymes with different preferences for the substrate as the other explanation for the additional metabolic effects seen in Fig. 3.

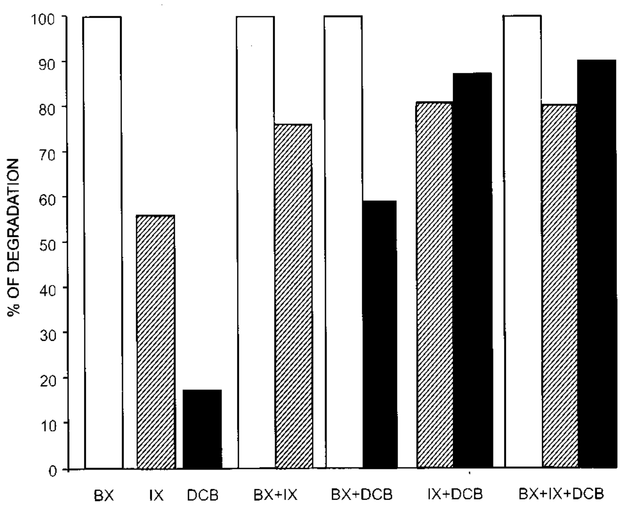

Fig. 3. Degradation of benzonitriles individually, and in their mixtures after 128 h of cultivation, by the strain *Agrobacterium radiobacter* 8/4. (Percentages of degraded benzonitriles are related to their initial concentration) (□) bromoxynil, BX, (▨) ioxynil, IX, (■) dichlobenil, DCB.

The low activity of nitrile hydratase in this study on dichlobenil alone may be explained by considering the chemical structure of this substrate. Two chlorines adjacent to the nitrile group may hinder binding to the enzyme in the case of dichlobenil, while bromoxynil and ioxynil have their halogenated substituents, bromine and iodine, respectively, in a distal position to the nitrile group which may be more conducive to binding.

3.3 Nitrile hydratase action on other aromatic nitriles

The range of *A. radiobacter* nitrile hydratase action on other aromatic nitriles is shown in Table 1. The data for the cell-free extract experiments indicate good activity of this enzyme on the substituted benzonitriles but not on indol-3-acetonitrile. Recently, Kobayashi *et al.* have reported isolation of a nitrile hydratase from *A. tumefaciens* acting on this substrate.[15] The authors, however, have predicted that various types of nitrile hydratase should be found in different *Agrobacterium* strains. This assertion seems to be confirmed by the finding that our strain possesses another type of nitrile hydratase than that found in *Agrobacterium tumefaciens.*

3.4 Conclusions

In our previous study we had determined that the degradation of halogenated benzonitriles leads to halogenated phenylamides.[9] These have been shown to be hydrolysed by various soil organisms with formation of the corresponding anilines which are further metabolized presumably through halogenated catechols.[16] Thus, the first step of degradation by nitrile hydratase seems to be crucial for the resulting fate of the herbicide mixture. From our data it is clear that in our conditions

TABLE 1
Degradation of Aromatic Nitriles by *Agrobacterium radiobacter* 8/4

Nitrile	*Degradation* by growing culture	*Degradation* by cell-free extract
2-Aminobenzonitrile	±	±
Benzonitrile	+	+
4-Bromobenzonitrile	+	+
Bromoxynil	+	+
3-Cyanopyridine	+	+
Dichlobenil	+	−
2,6-Difluorobenzonitrile	+	+
Indol-3-propionitrile	±	±
Indolylacetonitrile	−	−
Ioxynil	+	+
2-Hydroxybenzonitrile	±	±
3-Hydroxybenzonitrile	+	+
4-Hydroxybenzonitrile	+	+

the strain *A. radiobacter* 8/4 degrades bromoxynil most rapidly as a single substrate, while the best degraded mixture is that of bromoxynil with ioxynil.

More disputable is the interpretation of the data on inhibition of the *A. radiobacter* 8/4 growth rate. From this point of view, the most harmless single compound is dichlobenil, while the least inhibitive mixture is that of bromoxynil with ioxynil.

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